EFFECT OF THYROID HORMONES, SOMATOTROPHIN, INSULIN AND CORTICOSTEROIDS ON SYNTHESIS OF COLLAGEN IN GRANULATION TISSUE BOTH IN VIVO AND IN VITRO

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ABSTRACT

The effect of hormonal factors on the collagen synthesis in experimental granulation tissue of rats was studied (1) *in vivo* by assessing the development of the tensile strength and (2) *in vitro* by measuring the capacity of collagen synthesis from ^{14}C-proline by incubated granuloma slices. The hormonal agents were either administered *in vivo* or added to the incubation media.

When hormones were given *in vivo,* only cortisol affected the tensile strength of the granuloma markedly, but when the capacity of collagen synthesis was assessed, it was found that somatotrophin slightly increased and thyroxine decreased the synthesis. The effect of glucocorticoids, if any, was to increase the conversion of labelled proline into hydroxyproline.

In those experiments in which the hormonal factors were added to the incubation media, thyroxine and particularly insulin, increased the incorporation of ^{14}C-proline into collagen. Somatotrophin decreased the incorporation of proline in these experiments, presumably by artifact reactions.

The main purpose of the present experiments was to evaluate the effect of various hormonal factors on the synthesis of collagen in the experimental granulation tissue *in vitro.* As control, analogous experiments were made *in vivo* on the development of the tensile strength of the collagenous tissue in the granuloma. The hormonal factors included thyroid hormones, somatotrophin, insulin and glucocorticoids, which are all known to affect the metabolism of proteins, and particularly of collagen.

The effect of thyroxine or thyrotrophic hormone on the granulation tissue has been studied by *Saikku* (1956), *Koskinen* (1963), *Ther et al.* (1963), *Rodkina* (1964) and *Julesz et al.* (1964). The results have been conflicting. *Kivirikko et al.* (1963, 1964) studied the effect of thyroxine on the urinary excretion of hydroxyproline and observed an increase, but found little change in the content of collagen in the tissues.

Saikku (1956) and *Koskinen* (1963) demonstrated a favourable effect of somatotrophin on the development of the experimental granuloma.

The effect of insulin on the granuloma has been studied by *Nagy et al.* (1960) and *Rosenthal et al.* (1962), who observed a beneficial effect of insulin administration.

A good deal of attention has been paid to the effect of glucocorticoids. The entire literature cannot be mentioned here, but the general impression is that these steroids suppress the formation of collagenous tissue (*Lattes et al.* 1953; *Houck* 1962; *Ebert & Prockop* 1963; *Kivirikko* 1963; *Sandberg* 1963; *DiPasquale & Steinetz* 1964; *Nimni & Baretta* 1964; *Nocenti et al.* 1964), but *Jørgensen* (1962) found no effect. *Ther et al.* (1963) reported an increase in the tensile strength of the epiphyseal lines. The amount of salt-soluble collagen is decreased in glucocorticoid-treated animals (*Siuko et al.* 1959; *Kühn et al.* 1964) but the amount of insoluble collagen is not affected.

In spite of this wide interest, there is no information on the direct action of the hormonal factors on collagen synthesis. *Vaes & Nichols* (1962) studied the effects of hypophysectomy and of the administration of cortisone, thyroxine, insulin and oestradiol on the glycine incorporation in the organic matrix of incubated bone samples.

EXPERIMENTAL

Animals. Albino rats of Wistar-strain were used for the experiments both on the biosynthesis of collagen in granuloma slices (150–200 g) and on the tensile strength (140–160 g). The rats were kept in wire-bottom cages and fed with the standard laboratory diet *ad libitum,* except when stated otherwise.

Production of granulomata. Under ether-anaesthesia 2 or 4 standard pieces (10 × 10 × 20 mm, 60–90 mg dry weight) of viscose-cellulose sponges (from Säteri Oy., Valkeakoski, Finland) were implanted symmetrically under the dorsal skin of the rats. The sponge was sterilized previously by boiling for 10 min in 0.9 % NaCl-solution. For incubation experiments the rats were killed after 18–21 days. The granulomas were then prepared from the adjoining tissue, immersed immediately in cold 0.9 % NaCl-solution and cut into slices of 0.5 mm thickness (of 60–200 mg wet weight) with a Stadie-Riggs' microtome in the cold room. The slices were weighed with a torsion balance.

For the experiments of the tensile strength of the granulation tissue, similar pieces of the sponge were cut into two identical halves which were kept in the original location during growth by stitches (*Viljanto* 1964).

Measurement of tensile strength. The granulomas were harvested after 7 or 12 days. The tensile strength of the 7-day granulomas was measured according to *Viljanto et al.* (1962) and *Viljanto* (1964), but in the study of 12-day granulomas a larger balance (capacity of 2.5 kg) was necessary. The tensile strength of the skin wounds was determined on strips which had been cut (10 mm broad) perpendicularly to the wound as described by *Viljanto* (1964).

In the series of experiments in which the tensile strength of the developing granulation tissue was measured, treatment with hormones was initiated 3 days before the implantation and continued until the animals were killed, 7 or 12 days after the implantation. In all the experiments the control animals received 0.5 ml of 0.9 % NaCl-solution.

Hormonal preparations. The following agents were either administered *in vivo* in amounts mentioned in Tables 1 and 2 or added *in vitro* as listed in Table 3.

L-Thyroxine sodium 0.1 % solution in sterile water (Oy Star Ab, Tampere, Finland), given *in vivo*;

3,3',5-Triiodo-L-thyronine, purified grade (California Foundation for Biochemical Research, 3408 Fowler Street, Los Angeles 63, Cal.), added *in vitro*;

Somacton, »10 I. E. STH per ml pro inj. intramusc.« (Ferring Ab., Malmö, Sweden), given *in vivo*;

STH-substans (Lot. 3–63, Ferring Ab.), added *in vitro*;

Insulin Novo-Orion, 40 I. U./ml (from Oriola Oy, Helsinki, Finland), given *in vitro*;

Insulin Novo-Lente, 40 I. U./ml (from Oriola Oy, Helsinki, Finland), given *in vivo*;

Swine-insulin, 10 × cryst. (Lot. S 8563, Terapeutisk Laboratorium Novo, Copenhagen, Denmark), added *in vitro*;

Alloxanhydrat, 5,5-Dihydroxy-2,4,6-triketohexahydropyrimidine puriss. (No. 12698, Fluka AG, Buchs, Switzerland), given *in vivo*;

Actocortin, Natr. bis(21-hydrocortison)-phosphate amp. (Frederiksberg Chemische Fabriker; Oy Cortec Ab., Museok. 37, Helsinki, Finland), given *in vivo*;

Korti susp, Hydrocortisone acetate (Lot. 40114, Lääke Oy, Turku 17, Finland), given *in vivo*;

Prednisolone, Di-Adreson-F-aquosum (Prednisoloni natrii succinas lyophilisat, N. V. Organon-Oss, Holland), given *in vivo.*

Incubation of the slices. The incubations were carried out in flasks of the conventional Warburg constant volume manometers, at least in duplicate. The incubation solution was based on the work of *Green & Lowther* (1959) and contained the following final concentrations of the *L*-form of amino acids:

DL-Leucine (L. Light & Co, Ltd., Colnbrook, Bucks., England)	0.76 mM
L-Isoleucine (L. Light & Co, Ltd.)	0.38 mM
DL-Valine (S. A. F. Hoffmann-La Roche & Co, Ltd., Basel, Switzerland)	0.85 mM
DL-Threonine (Fluka AG, Buchs, Switzerland)	0.84 mM
DL-Methionine (E. G. Merck AG, Darmstadt, Germany)	0.13 mM
L-Arginine-HCl (E. G. Merck AG)	0.95 mM
DL-Lysine-HCl (E. G. Merck AG)	1.10 mM
L-Tyrosine (E. G. Merck AG)	0.22 mM
L-Glutamine (S. A. F. Hoffmann-La Roche & Co, Ltd.)	0.85 mM

The incubation solution was composed of the following parts (the final molar concentrations being as indicated):

9 ml of combined amino acid solution	
5 ml of NaCl-solution (4.5 %)	0.120 M
0.20 ml of KCl-solution 5.75 %)	0.005 M

0.15 ml of $CaCl_2$-solution (6.10 %) ..... 0.003 M
0.05 ml of $MgSO_4$-solution (19.10 %) ..... 0.001 M
5 ml of phosphate buffer (0.1 M, p_H 7.4) ..... 0.016 M
12.6 ml of water to make to the volume to 32 ml
129.0 mg of glucose was added as substance ..... 0.022 M

The hormones were added at the beginning of the incubation. The concentrations are shown in Table 3.

Incubation. Three ml of the above described incubation medium was pipetted into a Warburg flask and cooled to +4° C. One μc of ^{14}C-proline (The Radiochemical Centre, Amersham, Bucks., England) was added in 0.1 ml of aqueous solution. From six to eight slices, weighing altogether 800–1400 mg, were placed into flasks which were transferred to a Warburg apparatus. For the absorption of CO_2, 0.2 ml of 5 % potassium hydroxide was pipetted into the centre well. The data on the oxygen consumption were recorded only to ascertain if the conditions were right.

The usual incubation period was six hours after a 15-min preliminary warming. The incubation was ended by the addition of concentrated (1 g/ml) trichloroacetic acid solution (0.2 ml).

Determination of the radioactivities. The slices were washed and treated according to *Prockop et al.* (1961) for the separation of collagen-^{14}C-hydroxyproline and collagen-^{14}C-proline. The determination of ^{14}C-hydroxyproline was carried out according to *Peterkofsky & Prockop* (1962). The values for ^{14}C-proline refer to the first extract.

The radioactivities were measured with a scintillation head unit (NE 5503, Nuclear Enterprises Ltd., Edinburgh), connected through an amplifier with a scaling and high-voltage units (Frieseke & Hoepfner GmbH., Erlangen-Bruck, Germany). A high voltage of 675 V and an input voltage of 50 mV were found to be the most suitable.

Ten ml of the toluene solution which contained the extracted ^{14}C-derivatives of labelled proline and hydroxyproline were mixed with 1 ml of scintillation solution, composed of 15 g of 2,5-diphenyloxazole and 50 mg of *p*-bis-(2-(5-phenyl-oxazolyl)-benzene (both purchased from Fluka AG) in 1000 ml of redistilled toluene.

RESULTS

Table 1 shows the effects of the hormones given *in vivo* to rats during the period of growth of the granulation tissue. The effects are small, except after the administration of cortisol, which presumably inhibits primarily the proliferation of the fibroblasts. Alloxan in some instances by some obscure mechanism decreased the tensile strength, measured on the twelfth day.

The next step was to eliminate any complications due to the hormonal effects on cell proliferation. The hormones were administered *in vivo* shortly before killing the animals, the granuloma slices were then prepared and incubated *in vitro* in the presence of ^{14}C-proline. The formation of ^{14}C-hydroxyproline was accepted as a measure of the capacity for collagen synthesis. The supply of glucose and other substrates was the same for all the slices. The data are collected in Table 2. The experimental variation is considerable, but some results emerge. The activity of incorporated ^{14}C-proline varies in the control samples in the range of 4900–5700 cpm/100 mg of defatted dry tissue.

Table 1.

Effect of hormones on the development of the tensile strength in the experimental granulation tissue of rat.

The values are expressed in grams per standard granuloma piece or in grams per standard skin strip. *** $P < 0.001$, * $P < 0.05$. E experiment and C respective control. The number of the measurements is given in the brackets. The standard deviations are indicated.

Hormonal factor and tissue	Dose†	E/C	Interval after implantation	
			7 days	12 days
Thyroxine				
granulation tissue	500 μg/kg/day	E	86 ± 47 (9)	540 ± 120 (9)
		C	92 ± 40 (10)	561 ± 72 (10)
Somatotrophin				
granulation tissue	150 μg/rat/day	E	131 ± 63 (9)	578 ± 107 (9)
		C	122 ± 60 (10)	585 ± 100 (10)
Insulin				
granulation tissue	4 units/rat/day	E	80 ± 48 (8)	481 ± 69 (8)
		C	80 ± 32 (8)	503 ± 38 (9)
Alloxan				
granulation tissue	200–250 mg once intraperitoneally	E	132 ± 35 (11)	517 ± 77 (9)
		C	126 ± 33 (8)	508 ± 81 (9)
skin wound		E	209 ± 34 (12)	305 ± 64 (10)*
		C	206 ± 43 (10)	403 ± 131 (9)
Alloxan + insulin				
granulation tissue	alloxan as above, subsequently insulin 6 units/rat/day	E	106 ± 32 (16)	419 ± 74 (9)*
		C	98 ± 21 (8)	522 ± 77 (8)
Cortisone				
granulation tissue (hydrocortisone phosphate)	*7-day experiment* 25 mg/rat/day	E	23 ± 18 (10)	32 ± 40 (7)***
		C	80 ± 32 (8)	523 ± 80 (12)
skin wound (hydrocortisone acetate)	*12-day experiment* 25 mg/rat/day for 3 days, and then 2.5 mg/rat/day every other day	E	–	100 ± 25 (7)***
		C	–	403 ± 112 (8)

† treatment was initiated 3 days before the implantation and continued until killing of animals.

Somatotrophin causes an increase and thyroxine a decrease, which are suggestive, if not quite conclusive. With regard to hydroxyproline-^{14}C the control values vary in the range of 1400–1800 cpm/100 mg. Below this range are the

Table 2.

Effect of hormones, given *in vivo,* on the capacity of granulation tissue slices to synthetize collagen *in vitro.*

E experimental and C respective control values. The number of the experiments is given in the brackets. The activities of ^{14}C are calculated per 100 mg of dry defatted tissue.

Hormone	Dose	E/C	Incorporated amino acid				
			Proline-^{14}C		Hydroxyproline-^{14}C		Hydroxyproline-^{14}C / Proline-^{14}C
			cpm per 100 mg	% of control	cpm per 100 mg	% of control	
Thyroxine	500 μg/kg/day	E	3800 (2)	74	1100 (2)	78	0.29
	for 10 days	C	5100 (2)	–	1400 (2)	–	0.27
Somatotrophin	150 μg/rat/day	E	6700 (2)	131	2200 (2)	157	0.33
	for 10 days	C	5100 (2)	–	1400 (2)	–	0.27
Insulin	4 units/kg after	E	4200 (2)	85	1500 (2)	83	0.36
	24-h fasting	C	4900 (2)	–	1800 (2)	–	0.37
Alloxan	250 mg/kg once	E	5000 (3)	88	1800 (3)	100	0.36
	intraperitoneally	C	5700 (4)	–	1800 (4)	–	0.31
Alloxan+insulin	alloxan as above,	E	5300 (4)	93	2000 (4)	111	0.38
	insulin* 6 units/ day for 3 days	C	5700 (4)	–	1800 (4)	–	0.32
Prednisolone	40 mg/kg/day	E	5300 (2)	103	2100 (2)	150	0.39
	for 3 days	C	5100 (2)	–	1400 (2)	–	0.27

* for the first two days Insulin Novo-Lente and on the third day Insulin Novo-Orion.

values obtained by treatment with thyroxine, and above the results of treatment with somatotrophin and prednisolone, which both seemed to stimulate collagen synthesis under these conditions.

These data were supplemented by experiments, in which some hormones were added *in vitro* (Table 3). The results are derived from various experiments and are only comparable with the corresponding controls. Somatotrophin caused a sharp decrease in the synthesis, probably because of artifact reactions. The enhancing effect of thyroxine, even if small, should be noted (see Discussion). The most conspicuous effect is that of insulin: the synthesis of protein, and especially of hydroxyproline-containing protein, is increased.

DISCUSSION

There are certain discrepancies in the results obtained with various experimental procedures. Why is the effect of insulin not manifest, when it is given

Table 3.

Effect of added hormones on the capacity of granulation tissue slices to synthetize collagen *in vitro*.

E experimental and C respective control values. The number of the experiments is given in brackets. The activities of ^{14}C are calculated per 100 mg of dry defatted tissue.

Hormone and concentration in the medium	E/C	Incorporated amino acids				
		Proline-^{14}C		Hydroxyproline-^{14}C		Hydroxyproline-^{14}C / Proline-^{14}C
		cpm per 100 mg	% of control	cpm per 100 mg	% of control	
Tri-iodothyronine						
10^{-3} M	E	–	–	1200 (2)	100	–
10^{-4} M	E	–	–	1700 (3)	141	–
	C	–	–	1200 (6)	–	–
Insulin						
First experiment	E	23 600 (2)	100	2900 (2)	137	0.12
1 mg/ml	C	23 600 (2)	–	2100 (2)	–	0.09
Second experiment	E	12 600 (2)	161	2600 (2)	236	0.20
1 mg/ml	C	7 800 (2)	–	1100 (2)	–	0.14
Somatotrophin						
10 mg/ml	E	–	–	115 (1)	9	–
1 mg/ml	E	–	–	885 (1)	73	–
	C	–	–	1200 (4)	–	–

in vivo (Tables 1 and 2)? The animals were hypoglycaemic but the slices received an ample supply of glucose and amino acids. We believe that the prolonged hypoglycaemia could have damaged the cells.

The primary metabolic effect of the thyroid hormones seems to be a stimulation of protein synthesis (*Sokoloff et al.* 1963; *Tata et al.* 1963; *Tata* 1964). On this basis the data shown in Table 3 become more significant. The effects of thyroid hormone as shown in Table 2 may depend on secondary catabolic effects.

The general action of somatotrophin has been studied, among others by *Riggs & Walker* (1960) and *Korner* (1963) and enhanced transport of amino acids and stimulated synthesis of m-RNA have been implicated.

The most important finding in this study is the stimulating effect of insulin on the synthesis of collagen. Granulation tissue has not previously been observed to be an insulin-sensitive tissue (*Wool* 1964). We can only speculate as to the exact target of insulin, whether transport of substrates, generation of energy, modification of cellular structures or stimulated synthesis of RNA for

the synthesis of collagen are involved (*Wool* 1964). The next problem is to find out the conditions in which the beneficial effect of insulin can be exploited for the enhanced development of the tensile strength in healing wounds and in granulation tissue.

In general, the protein and nucleic acid synthesis can either be inhibited or stimulated by corticosteroids (*Feigelson & Feigelson* 1964; *Lang & Sekeris* 1964). From Table 2 it may be concluded that the capacity for collagen synthesis is not affected by glucocorticoids. It is well known that the administration of corticoids decreases the amount of soluble collagen, which seems to depend rather on suppressed metabolism of nucleic acids.

Attention should be paid to the varying ratio of hydroxyproline-^{14}C/proline-^{14}C in collagen (Tables 2 and 3). We do not know yet whether the proportion of hydroxylated amino acid in collagen is influenced, for example, by insulin and whether consequently the mechanical properties of collagen are qualitatively affected by hormones.

ACKNOWLEDGEMENTS

We are indebted to Sigrid Jusélius Foundation and U. S. Department of Agriculture, Foreign Research and Technical Programs Division, for institutional grants during this work. The somatotrophin preparations were a gift from AB. Ferring, Malmö. Sweden, by the courtesy of Dr. F. Paulsen. The thyroxine and insulin preparations were gifts from Messrs. Oy Star Ab, Tampere, and Messrs. Oriola Oy, Helsinki, respectively. For the viscose-cellulose sponges we are indebted to Messrs. Säteri Oy., Valkeakoski, Finland.

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